“Cow urine and its fractions as activity enhancer and availability facilitators for anti-malarial agent: Artemisinin”

SUMMARY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

Pharmaceutical sciences

By

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1. Chapter: Introduction

1.1 Introduction: ART is a good antimalarial drug but has a few limitations such as low bioavailability and failure to produce optimum effect on its administration alone. The ART belongs to BCS class II drugs with low solubility; hence its bioavailability is less. Moreover, its elimination half-life is also low. This affects its therapeutic potential. The combination of cow urine and its various forms may reduce these limitations with respect to ART in the management of malaria. Therefore, in the present study, more emphasis was kept on the use of a combination of ART and different forms of cow urine for the management of malaria. As an activity enhancer and bio-availability facilitator for bioactive compounds, including anti-infective and anti-cancer medicines, cow urine distillate has been granted a US Patent (US Patent No. 6410 059/2002). [1]

2. Chapter: Literature Review

2.1 Literature Review In this section the literature with respect to Malaria, its prevalence, severity, and management was covered. Then the literature regarding Artemisinin, Cow urine, and the development of formulation was mentioned.

3. Chapter: Research Envisaged & Plan of Work

3.1 Statement of the problem: Treatment of malaria becoming more and more complex due to the severe adverse effects of conventional drug therapy.

Development of resistance has become a challenge for the utilization of current anti-malarial drugs.

3.2 Conceptual framework and Rationale of the study: Owing to the serious adverse effects Treatment of active malaria is complex and challenging with conventional therapy. Moreover, only a small fraction of the anti-malarial drugs reaches the desired site of action leading to the prolongation of therapy at high therapeutic doses. This may lead to life-threatening complications, decreased bioavailability at the target sites, and patient non-compliance. Due to the costly and lengthy process of drug discovery and development, old drugs with enhanced therapeutic potential using bio-enhancer is emerged as an attractive strategy to provide rapid access to valuable anti-malarial medicines for patients.

3.3 Objectives:

- To evaluate Cow urine and its fractions as activity enhancers and availability facilitators for the Anti-malarial agent: Artemisinin
- To collect the cow urine and prepare various cow urine preparations.
- To assess the bioavailability potential of cow urine and its preparation for an anti-malarial drug: Artemisinin.
- To assess the in-vitro and in-vivo anti-malarial activity of cow urine and its preparations for an anti-malarial drug: Artemisinin.
- To prepare formulation and evaluation studies.

3.4 Work Plan and Research Methodology

- Collection of cow urine from nearby "Goshala."
- Characterization of fresh cow urine (pH, Specific gravity, Optical activity, etc.)
- Analysis of cow urine
- Development of various cow urine preparations.
- Qualitative and quantitative analysis of constituents of preparation mentioned above.
- Evaluation of the bio-availability potential of cow urine and its preparations mentioned above for the anti-malarial drug: Artemisinin.
- Selection of experimental animals.
- Estimations of various parameters
- Assessment of bio-enhancing activity of cow urine and its preparations for an anti-malarial drug: Artemisinin.
- Selection of experimental model (*In vitro* & *In vivo*)
- Estimations of various parameters
- Formulation and evaluation.

3.5 Expected outcome: Cow urine is commonly used in India. It was mentioned in traditional books that there are many health benefits of cow urine [2]. The present study was designed to carry out an evaluation of the bio-availability and anti-malarial activity-enhancing potential of cow urine and its preparations for an anti-malarial drug: Artemisinin.

4. Chapter: Materials and methods

4.1 Cow Urine preparations: Collection of Fresh Cow urine (FCU) [3], Preparation of Distillated Cow Urine (DCU) [4], preparation of The residue of cow urine [5], and Preparation of Fermented Cow Urine (FerCU) [6]

4.2 Qualitative analysis of different forms of cow urine [7]–[9]**:** Qualitative analysis was done by Preparation of Lassaigne's Solution (Sodium Filtrate), followed by Test for nitrogen, Test for sulphur, Test for chloride in FCU, DCU and RCU

4.3 Qualitative Examination of Inorganic and Organic Matters in FCU, DCU and RCU: Cow urine and its preparations were test for various biochemical components using conventional procedures[10].

4.4 Determination of pH and specific gravity different forms of cow urine: pH and specific gravity of FCU, DCU and RCU were determined using standard procedures [11],[12].

4.5 Preparation of formulation of Artemisinin along with Cow urine and its preparation using conventional method – "Bhavana"

Methods of Bhavana: Two techniques were used to carry out the bhavana process: lavigation and soaking (Nimajjana/Nivasana) [13]**.**

4.6 IR spectroscopy of the samples prepared by Bhavana method: samples made using the Bhavana procedure were measured using FT-IR equipment [14].

4.7 Toxicity studies

4.7.1 Acute oral toxicity studies: According to the OECD's 423 standard for testing chemicals, acute oral toxicity experiments were done for each of the eight formulations at 300 mg/kg (P.O) [15].

4.8 Selection of dose for animal studies

4.8.1 Dose of Artemisinin and formulations: 25 mg/kg of artemisinin was used in animal experiments[16], [17]. With respect to the ART normal animal dose of 25 mg/kg, the dose was chosen as 25 mg/kg.

4.9 UV spectroscopy analytical method development

4.9.1 Instrumentation: The absorbance was measured using a Perkin Elmer UV-VIS Spectrophotometer (Lambda 25) with matched quartz cells, a 10 mm optical path length, a set 1 nm spectral band width, and a 0.1 nm wavelength accuracy.

4.9.2 Drug solution: To give a concentration range of 2 µg/ml to 18 µg/ml, 1 ml of 0.25% NaOH was added to each dilution, followed by ethanol to volume make up the solution. The dilutions mentioned above were heated at 50℃ for 1 hour and then neutralised with 0.2 M citric acid.

4.9.3 Selection of wavelength of maximum absorbance (λ_{max}) of ART: The subsequent solution was then scanned under the UV range of 190 to 380 against reference solvent as blank.

4.9.4 Construction of Calibration curve of ART: The calibration curve was created at 256 nm in the reference solvent solution in the linearity range of 2 - 18 µg/ml.

4.9.5 Method validation: Validation of the developed method was done according to ICHQ2 (R1)guidelines for its specificity, linearity, accuracy, precision, LOD and LOQ [14-15].

4.9.6 Specificity: For the specificity investigation, the potential bias was assessed both with and without excipients using the overall percentage agreement.

4.9.7 Precision: The solution's absorbance (10 µg/ml) was measured six times for repeatability, and the results were recorded.

4.9.8 Repeatability: Determination of repeatability was done by analysing 10 µg/ml conc. of ART for 6 times.

4.9.9 Linearity and range: The linearity was determined by serial dilutions (2 to 18 µg/ml) of the ART in diluent.

4.9.10 Accuracy: The mean recovery should be desirably 100 ± 2% this was kept as accuracy criterion for this method at every concentration over the range of target concentration of 80-120%.

4.9.11 LOD and LOQ: The conc. of drug solutions series ranges from 2-10 µg/ml. LOQ &LOD values were calculated by the following equations.[20]

LOD = 3.3×N/B

LOQ =10×N/B

Where 'N' = SD of the peak areas of the drug (n=3), taken as measure of noise

B = slope of the corresponding calibration curve

4.10 *In-vivo* kinetic studies of samples prepared by lavigation and soaking method:

In this study, Adult male rats (n=5) weighing 250–350 g were used for *in vivo* kinetic studies, artemisinin and all other samples prepared by lavigation and soaking method namely ART+DCU (L), ART +FCU (L), ART +FerCU (L), and ART +RCU (L) of lavigated samples. ART+DCU (S), ART +FCU (S), ART +FerCU (S), and ART +RCU (S) of soaking samples were administered at a dosage of 25 mg/kg body weight (P.O).experimental conditions are mentioned in the following table no. 4.1

Table 4.1 Experimental conditions maintained for in-vivo kinetic study of samples prepared by lavigation and soaking method

Study design	**Cross over**
Number of groups	Number of groups: V
Number of animals in group:	Five
Selection of subject	Randomization
Study condition:	Fast state, Oral Pharmacokinetics
Reference product:	Artemisinin pure
Analytical method:	UV/Visible derivatization method
Analyte of interest:	Anti-malarial drug
Sampling Points:	5 (0.5hr. 1 hr. 2 hr. 4 hr. 6hr.)
Biological matrix:	Blood plasma
Method of sample collection:	Retro orbital vein
Washout period	≤ five half-lives of drug

4.11 In-vitro anti-malarial activity using Hemin inhibition assay

The -hematin inhibition assay was used to undertake in-vitro anti-malarial research following the completion of bioavailability experiments with all 8 samples.

4.12 Determination of Microbial content of residue of cow urine

The microbial content in the residue of cow urine was determined using method given in Indian Pharmacopoeia 2010. [21].

4.13 Formulation studies

Design, Characterization, Acute and Sub-Acute Toxicity studies and Pharmacokinetic Studies of Optimized Self-Emulsifying Nano Drug Delivery System (SNEDDS) incorporated with ART

4.13.1 Drug: ART gifted by Ipca laboratories Ratlam. All the remaining chemicals and reagents used in current study were of analytical grade.

4.13.2 Animals : In this study, healthy male Albino Wistar rats, weighing between 200 and 250g, were employed (IAEC, file number SPTM-IAEC/Feb-20/04/04).

4.13.3 Selection of oils*:* ART was quantified in different oils using a validated UV-spectrophotometer (Perkin Elmer Lambda 25) at 256 nm [22] and methanol as a blank. Readings were taken in triplicate [23].

4.13.4 Screening for surfactant and co-surfactant: Construction of pseudo ternary phase diagrams was done at different surfactant and co-surfactant ratios with different volume ratios of oil and Smix 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 respectively, to get maximum possible nano-emulsion regions [24].

4.13.5 Pseudo-ternary phase diagrams: To construct pseudo-ternary phase diagrams, values (end point) obtained from the titrations of homogenous mixtures of oil, Smix and water were used. The Smix was prepared in various ratios (1:1, 1:2, 2:1, 3:1, 4:1, 4:2 and 4:3). To construct a phase diagram for each Smix ratio – oil and a specific above mentioned Smix ratio were blended at different range of volume ratios like 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 [25], [26].

4.13.6 Formulation of ART-SNEDDS and ART+RCU (S) in SNEDDS: The ART-SNEDDS was made according to the region of nano-emulsion plotted in pseudo ternary phase diagrams obtained at different compositions. [27].

4.14 Characterization of ART-SNEDDS and ART+RCU (S) SNEDDS

4.14.1 Determination of droplet size, zeta potential and polydispersity index (PDI): The average droplet size and PDI of the reconstituted formulations were determined using photon correlation spectroscopy (PCS). [28].

4.14.2 Test for thermodynamic stability: The effect of temperature variation was also studied in order to determine thermodynamic stability of ART-SNEDDS and ART+RCU (S) SNEDDS using the heat- cool cycle, the freeze-thaw cycle and centrifugation. [29].

4.14.3 Visual Assessment of the formulation: In this study, the nature of formulations to self-emulsification was evaluated visually based on LFSC scale [30],[31]**.**

4.14.4 Dispersibility test / Time for self-nanoemulsification: Dispersibility and self-emulsification time was done by diluting 0.5 ml of both formulations in 500 ml of distilled water using USP apparatus II [32], [33].

4.14.5 Stability of ART-SNEDDS and ART+RCU (S) SNEDDS with respect to pH: They were visually observed drug precipitation and/or for phase separation in phosphate buffer pH 6.8, deionized water 6.4 pH and 0.1N HCl 1pH [34], [35].

4.14.6 % Transmittance: Samples were tested Using UV–visible spectrophotometer [36].

4.14.7 Transmission Electron Microscopy (TEM) Analysis of ART SNEDDS and ART+RCU (S) SNEDDS: ART-SNEDDS and *ART+RCU (S) SNEDDS* formulations were analyzed for their surface morphology using a TEM analysis at Diya labs, Mumbai. [37], [38].

4.14.8 In-vitro release studies ART SNEDDS and ART+RCU (S) SNEDDS

Samples were reconstituted in 1:20 ratio with water [39]. Further, they were placed in USP-II dissolution apparatus and measures using RP-HPLC at 256 nm [40].

4.14.9 Pharmacokinetics (In-vivo) studies: Healthy Wistar rats selected and grouped into 3 which receives ART suspension, ART-SNEDDS and ART+RCU (S) SNEDDS of 25 mg/kg. To get plasma, blood samples were taken at various intervals like 30, 60, 120, 240, 360 and 480 minutes. [41], [42].

4.14.10 Acute and Sub-Acute Toxicity studies of ART SNEDDS and ART+RCU (S) SNEDDS

4.14.10.1 The single dose acute toxicity study according to the OECD guideline 423: According to OECD guideline-423, three groups contained six animals at the dose 2000 mg/kg of the samples ART SNEDSS, and ART+RCU (S) SNEDDS were given orally. [43], [44].

4.14.10.2 Sub-Acute toxicity studies according to the OECD guideline 407: Biochemical parameters, urine analysis, Haematological parameters and Necroscopy: The repeated dose sub-acute oral toxicity study was performed according to the OECD guideline 407 [45]. A test substance was administered orally every day for 28 days at a dosage of 1000 mg/kg. [46].

4.14.11 MTT assay : MTT assay was performed to measure the cytotoxicity [47]. The J774 macrophage cells used in this experiment was done from the Deshpande Laboratories Pvt ltd, Bhopal, India. [48]. Percent viability was calculated by variation in absorbance of test samples and control samples.

4.14.12 Haemolytic toxicity: After mixing anticoagulant-treated rat whole blood with therapeutic doses of plain ART, ART SNEDDS, and ART+RCU (S) SNEDDS for 45 minutes at 37 0.5 °C, by Erythrocyte count and morphology studies were also performed on the blood samples to evaluate the percentage haemolysis [49].

4.15. *In-vitro* and *in-vivo* anti-malarial activity studies of ART SNEDDS and ART+RCU(S) SNEDDS

4.15.1 In-vitro anti-malarial activity -Maintenance of culture: *Plasmodium falciparum* MRC-2 strain was kept alive using the Trager and Jenson method [50].

4.15.1.1 Anti-plasmodial assay: Fluorescence was used to measure the *in vitro* anti-plasmodial activity of test materials 10uM in 0.1% DMSO against chloroquine sensitive and *Plasmodium falciparum* resistant strains [51].

4.15.2 *In-vivo* anti-malarial studies

4.15.2.1 Experimental animals: For *in vivo* anti-plasmodial activity tests, BALB/c mice (20–25 g) were employed. *P. falciparum* was intraperitoneally administered to four groups of six mice each and housed in different cages. Three groups each got 25 mg/kg of ART SNEDDS, ART+RCU SNEDDS and ART (the positive control).

4.14.7 Parasite infection of experimental animals: For the tests, *Plasmodium falciparum* was used. After confirming 20–30% parasitaemia in the donors. Animals were scarified and the contaminated blood was immediately drawn from the heart puncture site into a heparinized vacutainer tube. To reduce variability, the collected blood from all donor mice was pooled together and then diluted in sterile saline. After that, the

blood was diluted with physiological saline (0.9%) so that each 0.2 ml of the final solution contained roughly 1×10^7 parasitized red blood cells (PRBCs). Experimental animals received an intraperitoneal inoculation of 0.2 ml of diluted blood [52].

4.14.7 Test for suppressive activity (Peter's 4-day test)

Test of Suppression for Four Days The process that Peter et al. [26. An apparently healthy mouse from each group was coded and given 0.2 ml of blood containing roughly $1x10^7$ *P. falciparum*-infected RBCs on Day 0. Group 1 provided with 25 mg/kg of SnF-1 ART snedds, Group 2 receives 25 mg/kg SnF-2 ART RCU snedds, Group 3 received 25 mg/kg ART suspension and for Group 4 untreated control receives PBS. Treatment was administered from Day 0 to Day 3 during a four-day span. A sensitive digital analytical scale was used to assess each mouse's body weight on the first day (Day 0) and the fifth day (Day 4). Each experimental animal's tail blood was processed into a thin blood film on the fourth day (Day 4), preserved in methanol, and stained with Giemsa to reveal parasitized erythrocytes [53].

4.14.7 Determination of Parasitaemia and Survival Time: On the Day four, thin blood films were made by with drawing blood from each mouse's tail onto clean, non-greasy slides. After drying, the slides were viewed under a microscope using the 100 objectives.. Three fields, each with 200–500 cells, were counted on each slide. Parasitaemia was identified by employing a 100X objective lens in light microscopy and mentioned in following equation:

% Parasitaemia = (no. of parasitized RBC / Total no. of RBC counted)×100

5. Chapter: Result and discussion

5.1 Identification and Characterization of Artemisinin: Artemisinin was procured from IPCA laboratories Ratlam as a gift sample.

5.1.1 Solubility studies: Studies were done according to the solubility chart provided by the I.P. and it was found that ART is practically in-soluble in water.

5.1.2 Melting point determination (9): The temperature at which melting first started to occur was 152 °C, and melting continued until it reached 161 °C [54].

5.1.3 Identification of Artemisinin by FT-IR: The FT-IR spectra of artemisinin were determined using the FT-IR apparatus for the purpose of confirmation of purity of the drug. Results are shown in Figure 5.1 and Table 5.1 [55].

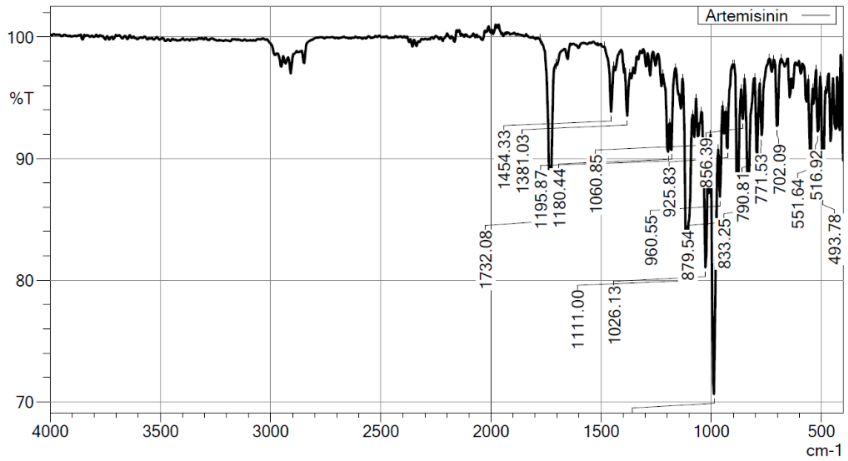


Figure 5.1. FT-IR of Artemisinin

Table 5.1 FT-IR interpretation of Artemisinin

S.no	Functional group	Theoretical wave no. (cm-1)	Observed wave no. (cm-1)
1.	C=O	1730 - 1750	1732.08
2.	C-H bending	…….	1180
3.	O-H over tone	1450	1454.33

5.1.4 HPLC analysis of Artemisinin[56]

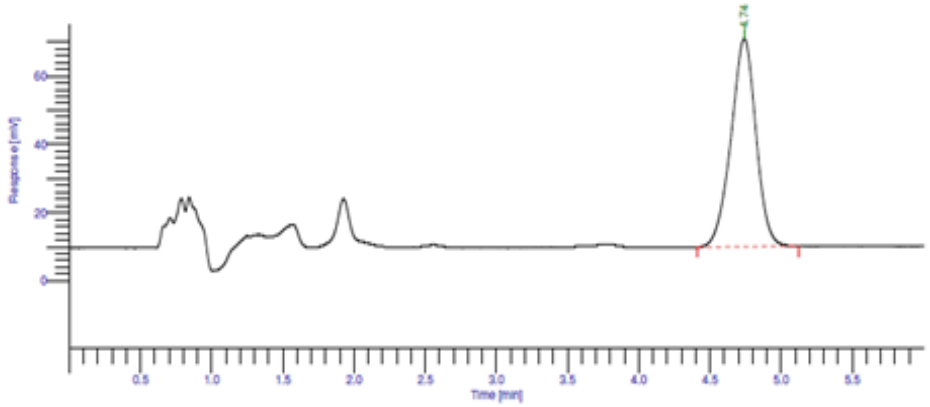


Figure 5.2 HPLC analysis of Artemisinin

Table 5.2 Chromatographic Conditions

Drug	Column	Mobile phase	Oven temperature	Injection volume	Flow rate	Retention time	Wavelength (nm)
Artemisinin	c18 column 250 mm x 4.6 mm x 5u	Acetonitrile : water (60:40)	25°C	20 µl	1m L min −1	15 min	209 nm

As per the data obtained in FT-IR and HPLC analysis, the test sample was confirmed to be ART (Fig 5.2 and Table 5.2).

5.2 Collection of Fresh Cow urine (FCU): The amount of fresh cow urine collected at a single time was found to be approximately 1 liter (Fig 5.3). [57].

5.2.1 Preparation of Distilled Cow urine (DCU): The amount of distilled cow urine obtained after the process of distillation was found to be 920 ml to 970 ml approximately (Fig 5.3).

5.2.2 The residue of cow urine: Yield for 1 lit of FCU after the distillation process was found to be: 40-50 gm. of Residue (Fig 5.3) [5].

5.2.2.1 Preparation of fermented cow urine: After 21 days of fermentation of Cow urine along with old jaggery, we got the Fermented cow urine (FerCU). Which changes its color into dark brown (Fig 5.3) and developed a unique smell

Figure 5.3 Cow urine and its preparations

5.2.3 Qualitative analysis of FCU, DCU, RCU and FerCU [58]**.**
The results of the elemental analysis of FCU, DCU, RCU, and FerCU are given in Table 5.3.

Table 5.3 Elemental analysis of FCU, DCU, RCU, and FerCU

Test for elemental analysis	Nitrogen urine (Ganvati)ion		Sulphur		Chloride	
	Observation	Inference	Observation	Inference	Observation	Inference
FCU	Blue color	Present	violet color	Present	White precipitate	Present
DCU	Blue color	Present	violet color	Present	No White precipitate	Absent
RCU	No blue color	Absent	No violet color	Absent	White precipitate	Present
FerCU	Blue color	Present	violet color	Present	White precipitate	Present

5.2.4 Qualitative Examination of Inorganic and Organic Matters in FCU, DCU, RCU and FerCu

Table 5.4. Qualitative test for chemical composition of FCU, DCU, RCU and FerCU.

S.NO.	Component	FCU	DCU	RCU	FerCU
1	Urea	P	A	A	P
2	Potassium	P	P	A	A
3	Calcium	P	A	P	P
4	Iron	P	A	A	A
5	Phosphorus	A	A	A	A
6	Bi-carbonate	P	P	A	A
7	Sulphate	P	A	A	A
8	Bile pigment	P	A	P	P
9	Carbohydrate	P	P	P	P
10	Protein	P	A	P	A
11	Ketone bodies	A	A	A	A
12	Creatinine	P	A	A	P
13	Ammonia	P	P	P	P
14	Uric acid	P	P	P	P
15	Tartaric acid	P	P	P	P
16	Oxalic acid	P	P	P	P

(A: Absent, P:Present)

Table 5.5 Determination of pH and specific gravity of FCU, DCU, and RCU [59]**.**

S.No.	Cow urine & preparations	pH	Specific gravity
	Fresh cow urine	7.82±0.05	1.027±0.13
	Distillate cow urine	8.82±0.07	0.997±0.08
	Residue of Cow Urine	10.0±0.11	1.035±0.16

5.3 Preparation of formulation of Artemisinin along with Cow urine and its preparation using the conventional method – "Bhavana"

The Bhavana process is of two types lavigation method and the soaking method. A list of the samples prepared by using both methods separately are mentioned below (Figure 5.4). List of the samples prepared are ART + FCU (L), ART + DCU (L), ART + FerCU (L), ART + RCU (L), ART + FCU (S), ART + DCU (S) , ART + FerCU (S), and ART + RCU (S).

Note: *ART: Artemisinin, *FCU: Fresh Cow Urine, *DCU: Distillate Cow Urine, *FerCU: Fermented Cow Urine, *L: Lavigation, *S: Soaking method

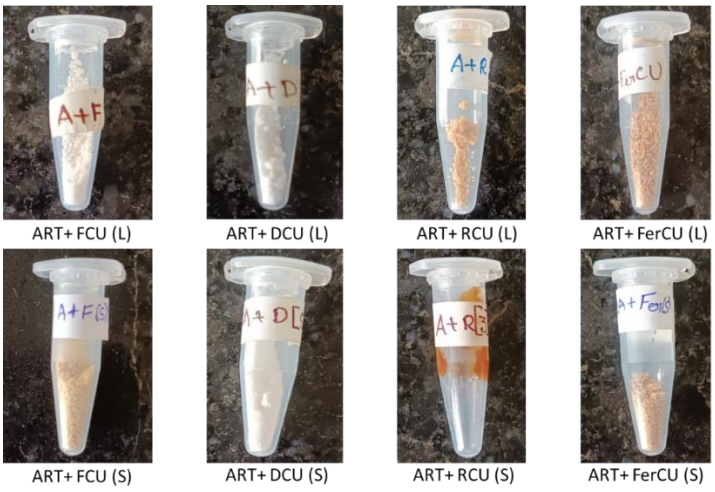


Figure 5.4 Formulations prepared by Lavigation and Soaking method

5.3.1 IR spectroscopy of the samples prepared by Bhavana method.

Representative graph of sample 8. FT-IR of ART+ RCU (S)

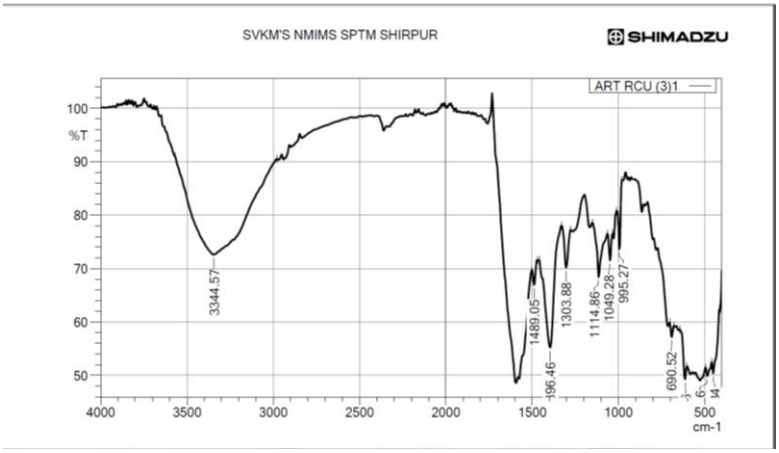


Figure 5.5 IR of Sample 8 ART+ RCU (S)

Table 5.6 IR Interpretation of ART+ RCU (S)

S.no	Functional group	Theoretical wave no. (cm-1)	Observed wave no. (cm-1)
1.	O–H stretch, hydrogen bonded	3500-3200 cm-1	3300.57
2.	C=O	1730 - 1750	1732.08
3.	O-H over tone	1450	1454.33
4.	C-H bending	…….	1180

According to the IR interpretation of above graphs, the samples made via the Bhavana method does not change the ART and retained it as such along with the cow urine and its derivatives.

5.4 Toxicity studies

5.4.1 Acute oral toxicity studies: Test samples show no signs or symptoms of toxicity and no mortality rate was observed at 2000 mg/kg.

5.4.2 Selection of dose for animal studies

5.4.3 Reported Dose of Artemisinin: According to reports, experimental mice were given oral doses of Artemisia annua extracts ranging from 1000 to 5000 mg/kg without

experiencing any apparent acute adverse effects [60]. Artemisinin dose for animals was reported as 25 mg/kg [61].

5.4.4 Selected Dose of Formulations: No animal exhibits hazardous symptoms during the 14-day observation period at 2000 mg/kg. With respect to the ART normal animal dose of 25 mg/kg, the dose was chosen as 25 mg/kg.

5.5 UV spectroscopy analytical method development

The developed method was simple, rapid, acid stabilized and Bathochromic shift-based UV spectrophotometric technique was validated for determination and estimation of ART.

5.5.1 Determination of wavelength of maximum absorbance(ʎmax) of ART: The absorption spectra of standard solution of 2 µg/ml of ART was scanned in 190 to 380 nm range against reference solvent as blank. The maximum wavelength of ART was found at 255.6≅ 256 nm. (Table 5.16, Figure 5.6).

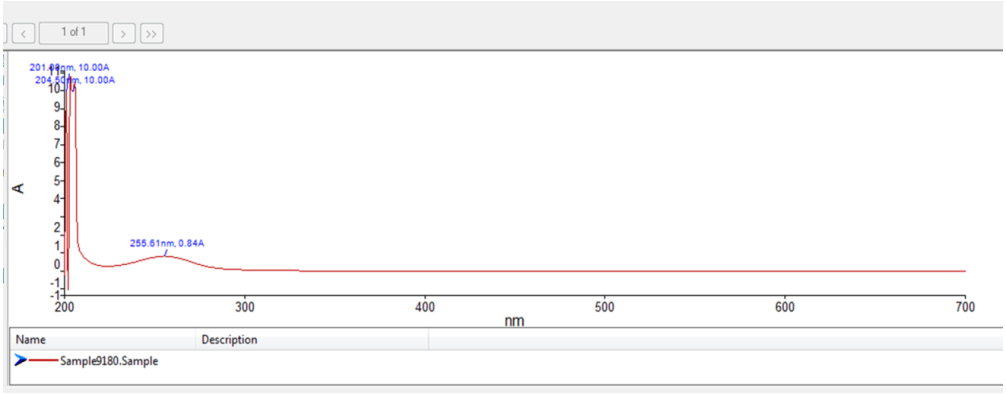


Figure 5.6 UV-Visible spectra of 2 µg/ml of standard ART solutions in solvent at 256.6nm.

5.5.2 Calibration curve of ART: At optimum experimental conditions, a calibration curve was plotted as concentration versus absorbance in range of 2-18 µg/ml (Table 5.7). High value of correlation coefficient (0.9928) indicates good linearity and adherence to Beer's law. Beer's law data and regression characteristics were determined (Table 5.17 and 5.18 & Fig 5.7).

Table 5.7 Calibration curve data for ART.

Conc. (µg/ml)	2	4	6	8	10	12	14	16	18
Abs*	0.189	0.251	0.354	0.442	0.541	0.633	0.677	0.754	0.820

***** Data represents mean of triplicate determinations.

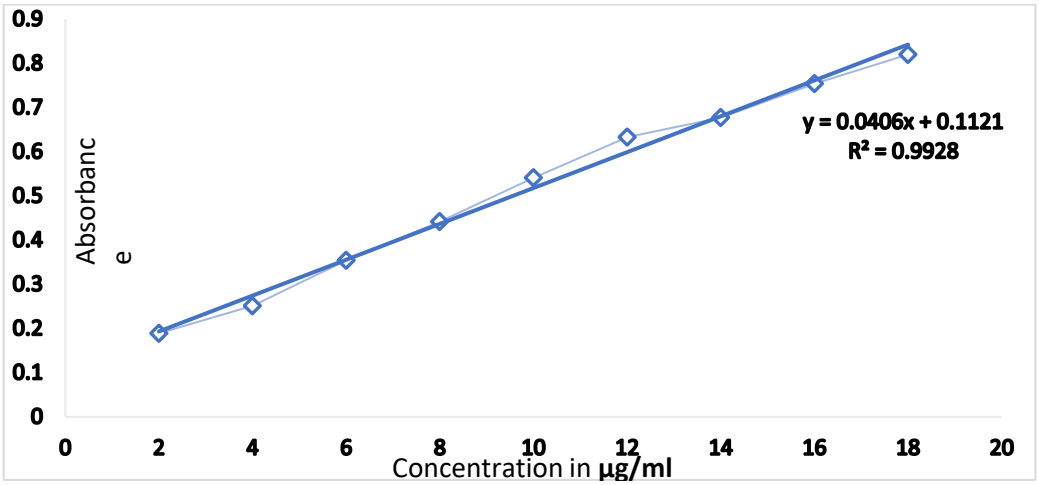


Figure 5.7 Calibration Curve of ART at 256 nm.

5.5.3 Specificity: The placebo sample has not shown any potential interference in absorption spectra of ART formulation at λmax of 256 nm. The % Agreement was shown 104.112.

% Agreement = TP/TA×100

TP = test result in the presence of placebo excipients

TA = test result in the absence of placebo excipients

5.5.4 Precision: The method shows good repeatability that was demonstrated by RSD (Relative Standard Deviation) <2%. The RSD was 0.247% (Table 5.8).

5.5.5 Linearity and range: Regression analysis was made for intercept, slope, and correlation coefficient value using the method of least square. (Table 5.8).

Table 5.8 Data of Beer's law and regression characteristics.

Parameters	**UV spectrophotometric methods**
Regression equation	Y = 0.040x + 0.112
Slope (b)	0.0406
Intercept (a)	0.1121
Linearity range (mg /ml)	2–18
Molar absorptivity (Lit/mole/cm)	8.12×282.332=2286.88
Correlation Coefficient (r)	0.9928

5.5.6 Accuracy: The developed method was evaluated for its accuracy, based on recovery studies. The mean recovery should be desirably 100 ± 2% this was kept as accuracy criterion for this method at every concentration over the range of target concentration of 80-120%. (Table 5.9). The % recovery differs from 99.3% to 101.30%.

5.5.7 LOD and LOQ: The developed method was highly sensitive to detect and determine the ART content. The LOD for S/N ratio of 3:1 was 0.106 µg /ml and the LOQ for signal to noise ratio of 10:1 was 0.322 µg /ml. the data was depicted in Table 5.9.

Table 5.9 Method validation of various parameters of method developed for ART.

Parameters	**Values**		
Linearity(mg/ml)	2-18 mg/ml		
Correlation coefficient	r^2 = 0.9928		
Accuracy (% Recovery)	99.3-101.30%		
Precision			
Repeatability	(% RSD) <0.247		
Intraday	0 hr (% RSD) 0.816	4 hr (% RSD) 0.329	8 hr (% RSD) 0.094
Inter-day	Day 1 (% RSD) 0.4631	Day 2 (% RSD) 0.4389	Day 3 (% RSD) 0.605103
LOQ (mg/ml)	0.322		
LOD (mg/ml)	0.106		
Specificity	104.112 (Avg agreement %)		

5.6 *In-vivo* kinetic studies of samples prepared by lavigation and soaking method

Preclinical pharmacokinetic studies of the formulations made from ART and cow urine and its preparations show significantly better results when compared to pure ART monotherapy in parameters like Cmax: maximum concentration of drug absorption was seen with ART+RCU(L) of 7.7±0.41*** mcg/ml and ART+RCU(S) of 9.9±0.76*** mcg/ml when compared to ART pure of 2.9±0.32 mcg/ml and 2.6±0.29. There were no significant difference observed in t_{max} of all the samples. Where as $t_{1/2}$ of ART+RCU(L) & ART+RCU(S) was raised to 159±17*** & 142±6*** minutes, respectively. All the results are represented in the following tables 5.10 and 5.11 Dose of the all the samples was 25 mg/kg.

Table 5.10 Pharmacokinetics studies of test samples prepared by lavigation method (on oral administration) using kinetica software

Parameters	**Group-I**	**Group-II**	**Group-III**	**Group-IV**	**Group-V**
C_{max} in mcg/ml	2±0.29	3.3±0.29	7.7±0.41***	2.1±0.15	2.9±0.32
T_{max} in Min	160±25	200±25	220±20	160±25	200±25
AUC_{last} (Min)*(mcg/ml)	66±7	75±9.8	212±57**	60±8.9	57±8.3
AUC_{tot} (Min)*(mcg/ml)	120±5	146±26	540±27***	111±14	120±15
t_{half} in Min	88±3.2	87±6.3	159±17***	90±3.6	99±4.5

All the results expressed as mean±SEM and Statistical analysis was done using One Way ANOVA followed by Dunnett's Multiple Comparison Test, where ($P < 0.05$) is considered as significant as compared to Group V.

Table 5.11 Pharmacokinetics studies of test samples prepared by soaking method (on oral administration) using kinetica software

Parameters	Group-I	Group-II	Group-III	Group-IV	Group-V
C_{max} in mcg/ml	2.5±0.37	4.5±0.5*	9.9±0.76***	2.8±0.29	2.6±0.29
T_{max} in Min	140±20	180±27	240±0	200±25	180±27
AUC_{last} (Min)*(mcg/ml)	160±24	185±15	383±23***	167±11	157±13
AUC_{tot} (Min)*(mcg/ml)	266±18	347±29	538±21***	276±43	323±24
t_{half} in Min	75±2.9	91±4.6*	142±6***	91±5.1*	71±6.2

All the results expressed as mean±SEM and Statistical analysis was done using One Way ANOVA followed by Dunnett's Multiple Comparison Test, where ($P < 0.05$) is considered as significant as compared to Group V.

5.7 *In-vitro* anti-malarial activity

5.7.1 Hemin inhibition assay: In this study, the antimalarial activity of 8 test samples was assessed *In-vitro*. The sample ART+RCU (S) had considerable potential antimalarial effects, with an IC_{50} value of 222.772 27.76 (12.46%) Figure 5.8 ART+RCU (S) was chosen for additional research on formulation, characterization, oral kinetics, and *In-vitro* and *In-vivo* anti-malarial activity (Table 5.12).

Table 5.12 *In-vitro* anti-malarial activity and IC_{50} of samples prepared by lavigation method and soaking method using Hemin inhibition assay.

Groups	IC50 Value in µM
I-ART+FCU(L)	458.42±26.67
II-ART+DCU(L)	468.45±31.01
III-ART+RCU(L)	387.07±35.67
IV-ART+FerCU(L)	520.78±86.88
V-ART+FCU(S)	390.92±27.05
VI-ART+DCU(S)	399.13±10.64
VII-ART+RCU(S)	222.77±27.76**
VIII-ART+FerCU(S)	408.88±82.58
IX-ART	548.57±56.7

IC_{50} Valuses of different samples by Hemin Inhibition Assay

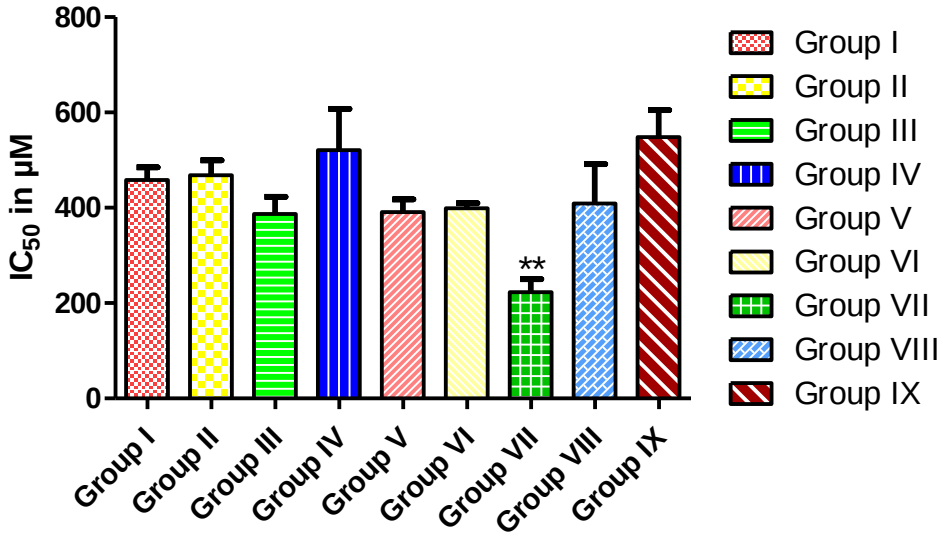


Figure 5.8 IC50 values of different samples by Hemin inhibition assay

All the results were expressed as mean±SEM and statistical analysis was carried out by one way ANOVA followed by Dunnett's Multiple Comparison Test, where ($P < 0.05$) is considered as significant as compared to Group IX.

5.8 Microbial content of residue of cow urine: The determination of microbial content in the residue of cow urine was carried out using CFU method. In this study it was observed that only one colony was found in petri plate containing 10 µl of residue of cow urine where as in petri plate containing 20 µl there were two colonies were observed. In the present study it has been found that the plate containing 10 µl test sample has shown only one colony while in plate containing 20 µl test sample shown two colonies. Thus, the developed colonies in both the plates are within the limits.

5.9 Formulation studies

Design, Characterization and Pharmacokinetic Studies of Optimized Self-Emulsifying Nano Drug Delivery System (SNEDDS) incorporated with ART

According to the literature and physicochemical properties of the ART the attempt was made to develop the ART SNEDDS as a novel formulation.

5.9.1 Selection of oils: Further, in this study, it has been seen that the solubility of ART was greater in soybean oils as compared to the other oils obtained naturally. The ART was 98.251 % solubilized in soybean oil. Thus, soybean oil was used for the formulation of ART-SNEDDS. The results are depicted in Table 5.13 and Figure 5.9.

Table 5.13 Solubility of ART in different oils

Olive oil	Coconut oil	Soybean oil	Peanut oil	Linseed oil	Castor oil	Corn oil
48.49%	94.31%	98.25%	70.66%	47.01%	28.79%	49.23%

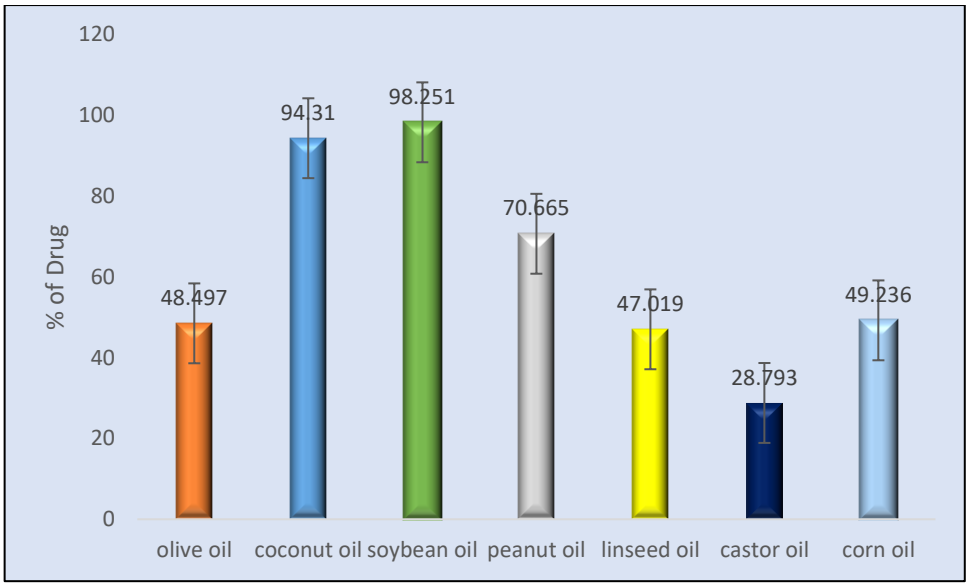


Figure 5.9 Solubility of ART in different oils

5.9.2 Surfactant and co-surfactant Selection for ART-SNEDDS preparation: According to this study the tween 80 has shown more oil solubilizing ability i.e., 1.36 w%. Hence, the further study was continued using tween 80. In this study the soy lecithin was used for the development of ART-SNEDDS as the co-surfactant. The nano-emulsion

formation was taken place as there is a reduction in free energy. The results are given in Table 5.14 and Figure 5.10 [62].

Table 5.14 Solubility of ART in different oils

Span 20	Tween 20	Tween 80	Soy lecithin
0.7 w%	0.9 w%	1.36 w%	1.14 w%

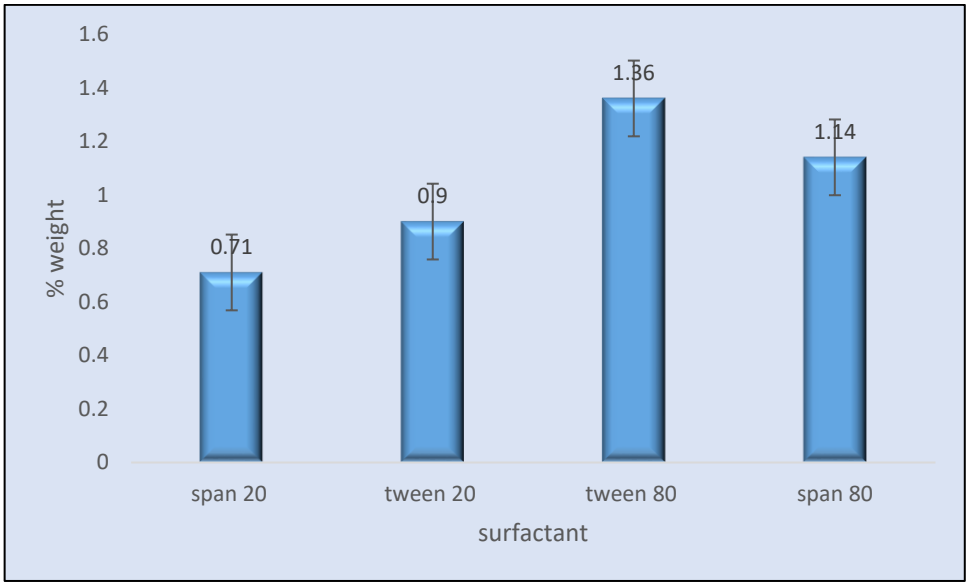


Figure 5.10 Oil solubilized (weight %) by different surfactants

5.9.3 Construction of pseudo ternary phase diagram: The area of the nano-emulsion region was found by the construction of pseudo-ternary plots. [63]. The Figure 5.11 has shown different diagrams (pseudo-ternary phase) of the various systems comprising soybean oil, Tween 80 as surfactant and soy lecithin as co-surfactant, when used in various ratios (around 7 ratios of Smix). In the Figure 5.11 the ratio of Smix was 4:3 which has shown wide naon-emulsion region. Moreover, as the ratio changed from 4:3 to 4:2 the emulsion area got decreased. Nevertheless, change in the Smix ratio as 3:1, 2:1, 1:2, and 1:1 leads to further reduction in nano-emulsion area more considerably. Furthermore, the increase in nano-emulsion area when Smix ratio was 4:3 owing to the improved surfactant molecules adsorption at the interface of oil and water. Amongst, the combination containing amount of oil 34%, 44 % water and 23 % Smix of ratio 4:3 was chosen for the preparation of ART-SNEDDS based on the pseudo ternary phase plots. The titration table for construction of Pseudo ternary phase diagram for the Smix ratio

4:3 was depicted below in Table 5.15. The ratio 1:20 was used with water to dilute the formulation to observe visually for grading.

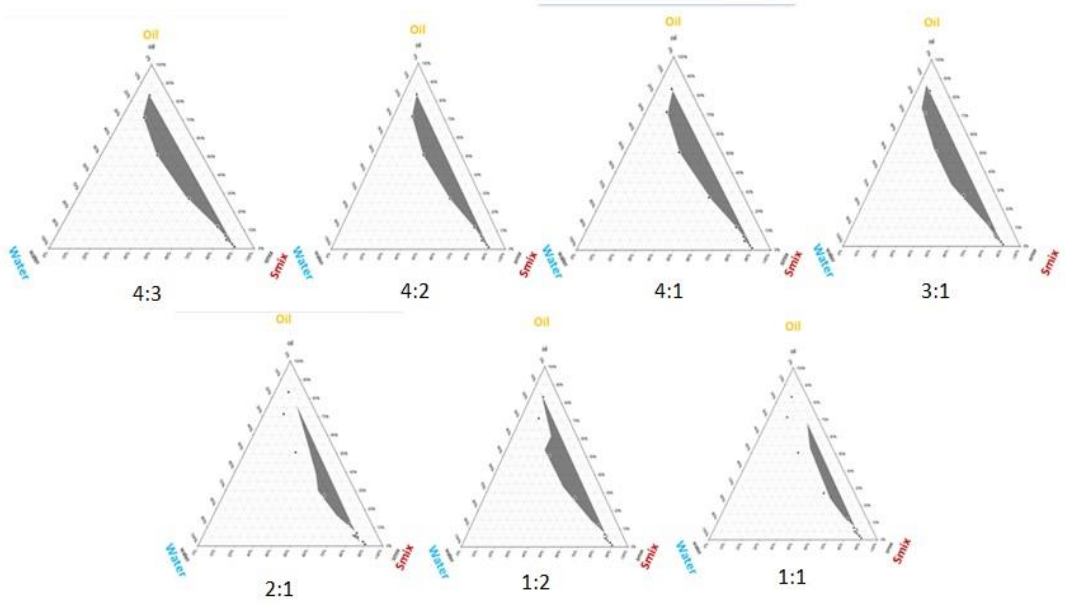


Figure 5.11 Oil and Smix Ratio selection based on Pseudo ternary phase diagram

Table 5.15 The titration table for construction of Pseudo ternary phase diagram for the Smix ratio 4:3

Formulation Number	Oil (%w/w)	Smix (%w/w)	Water (%w/w)
1	1.09	9.79	89.14
2	2.57	10.26	87.18
3	5.18	12.07	82.76
4	5.48	8.22	86.32
5	13.16	13.16	73.69
6	24	16	60
7	**34**	**23**	**44**
8	53.34	13.34	33.34
9	68.19	7.58	24.24

5.10 Characterization of ART-SNEDDS and ART+RCU (S) SNEDDS: The characterization of the developed ART-SNEDDS and ART+RCU (S) SNEDDS was carried out and the details are given in the following subsections.

5.10.1 Determination of Polydispersity index (PDI), Droplet size and zeta potential

The average globule size of ART-SNEDDS, ART+RCU (S) SNEDDS was determined to be 100 nm - 200 nm which was in nanometric range. ART SNEDDS, ART+RCU (S) SNEDDS displayed a limited size distribution with a PDI value of 0.315±0.004 and 0.203±0.002 respectively. Results are depicted in following Table 5.16.

5.10.2 Test for thermodynamic stability: The ART-SNEDDS and ART+RCU (S) SNEDDS form thermodynamically stable nanoemulsion system when undergoes in situ solubilization at 4ºC and 45ºC in three heating and cooling cycles and at -20ºC and 25±2ºC in three freeze-thaw cycles, without any sign of cracking, creaming and/or phase separation. In the Table 5.16. all results with respect to thermodynamic stability studies are tabulated.

5.10.3 Visual Assessment of the formulation: Visual assessment of formulation ART-SNEDDS and ART+RCU (S) SNEDDS shown that the formed emulsions were physically stable after several weeks of dilution with water based on LFCS table. (Table 5.16). [64].

5.10.4 Dispersibility test / Time for self-nano emulsification: Tween 80's greater ability for solubilization was shown to emulsify the formulation in less time than a minute with clear emulsion and based on dispersibility grade Table 4.8 formulation was classified as Grade A [65].

5.10.5 Stability of ART-SNEDDS and ART+RCU (S) SNEDDS with respect to pH

After dilution, there were no indications of drug precipitation and phase separation found with both the formulations.

5.10.6 % Transmittance: An important factor in understanding, if a system is isotropic, is its % transmittance. The ART-SNEDDS and ART+RCU (S) SNEDDS % transmittance value was found to be more than 90%.

Table 5.16 characterization of optimized ART-SNEDDS and ART+RCU (S) SNEDDS formulation containing selected oil (soy bean), selected Smix [tween 80: soy lecithin (4:3)], and water (4:2:4) respectively.

Formulation characterization parameter	**Result**	
Visual assessment	**ART-SNEDDS**	***ART+RCU (S) SNEDDS***
Spontaneity	Efficient	Efficient
Homogeneity	Yes	Yes
Dispersibility (time)	< 15 sec	< 15 sec
Appearance	Bluish transparent	Bluish transparent
Thermodynamic stability studies for drug loaded formulations		
Centrifugation	Passed	Passed
Freeze-thaw cycle	Passed	Passed
Heating cooling cycle	Passed	Passed
Dispersibility test		
Dispersibility & appearance	A clear nano-emulsion was formed quickly	A clear nano-emulsion was formed quickly
Time for self emulsification (min)	< 1 min	< 1 min
Grade	A	A
Stability of ART-SNEDDS with respect to pH	Pass	Pass
% transmittance	92.53±0.21	91.91±0.20
Mean droplet size	100 nm	100 nm
Poly dispersity index	0.315±0.004	0.203±0.002
Zeta potential	44.43±0.12	56.50±0.12

5.10.7 Transmission Electron Microscopy (TEM) Analysis of ART SNEDDS and ART+RCU (S) SNEDDS: The results of droplet size of the ART-SNEDDS and ART+RCU (S) SNEDDS were summarized in Figure 5.12, 5.13 & table 5.16. The ART-SNEDDS and ART+RCU (S) SNEDDS formulations has a PDI value of 0.315±0.004 and 0.203±0.002 respectively and a size of 100 nm, demonstrating the high stability of Nano formulation

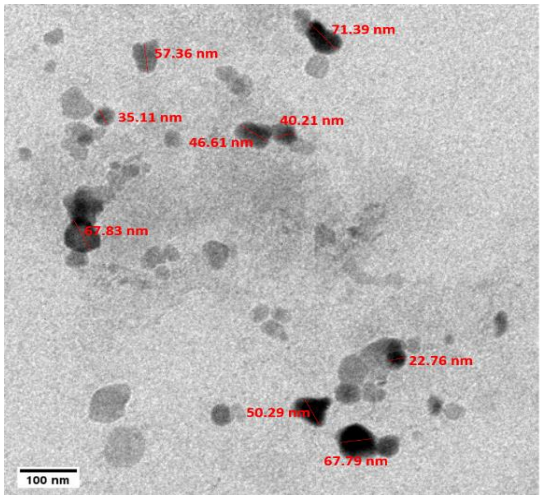


Figure 5.12 TEM analysis of ART-SNEDDS

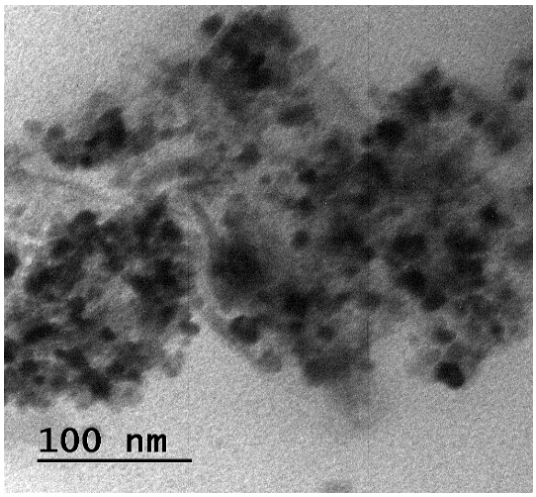


Figure 5.13 TEM analysis of ART+RCU(S)-SNEDDS

5.10.8 *In-vitro* release studies

In-vitro drug release studies were shown in figure 5.14. When compared to ART suspension, the drug release from ART-SNEDDS and ART+RCU (S) SNEDDS formulations was found to be remarkably significant ($p < 0.01$). This formulated ART-SNEDDS and ART+RCU (S) SNEDDS carried forward for *In-vivo* kinetic studies since they showed increased drug release of ≈ 99.14 % in *In-vitro* release studies, where ART suspension shows only 50.09 %.

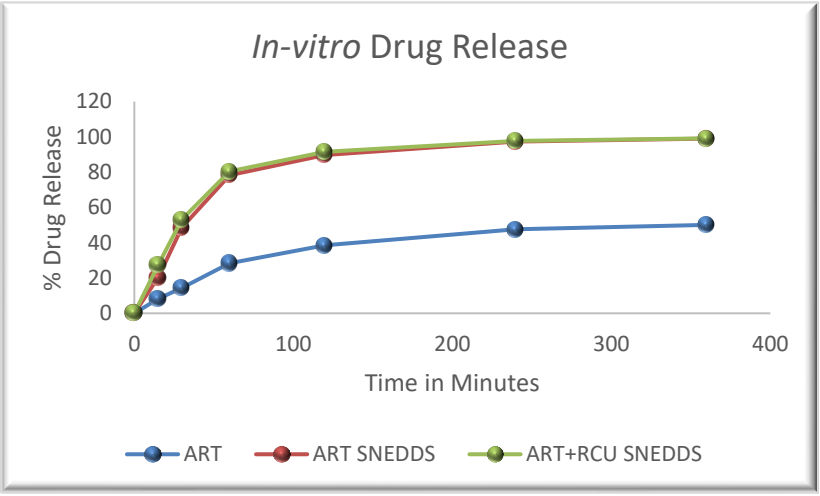


Figure 5.14 *In-vitro* release studies of ART suspension, ART-SNEDDS and ART+RCU (S) SNEDDS

5.10.9 Pharmacokinetic (*In-vivo*) studies

Oral bio-availability studies of ART-SNEDDS and ART+RCU (S) SNEDDS shown a significant increase in absorption of drug and total plasma concentration at every sampling point ($p<0.01$) when compared to ART suspension (Figure 5.25, Table 5.17.). And hence, the study reflects the developed ART-SNEDDS and ART+RCU (S) SNEDDS formulation have a good impact on increasing oral absorption of ART. This increased absorption of ART in SNEDDS might be due to the reduced particle size less than or equal to 100 nm. Therefore, it can be concluded that the developed ART SNEDDS and ART+RCU (S) SNEDDS formulation could overcome solubility issues, which are a major drawback of ART.

Table 5.17 Pharmacokinetics studies of test samples (on oral administration) using Kinetica software

Parameters	ART	ART SNEDDS	ART+RCU SNEDDS
C_{max} in mcg/ml	2.15±0.8	4.9±0.34***	7.42±0.25***
T_{max} in Min	60±0.68	60±0.61	120±0.81***
AUC_{last} (Min)*(mcg/ml)	414.8±0.3	701.88±0.20***	1769.4±0.19***
AUC_{total} (Min)*(mcg/ml)	452.75±1.32	768.37±1.19***	2419.38±0.94***
t-half in Min	51.61±1.94	58.64±1.51	116.15±1.14

The results were espressed as mean±SEM and Two Way ANOVA followed by Bonferroni multiple comparison test was used for statistical analysis where *** P<0.001)

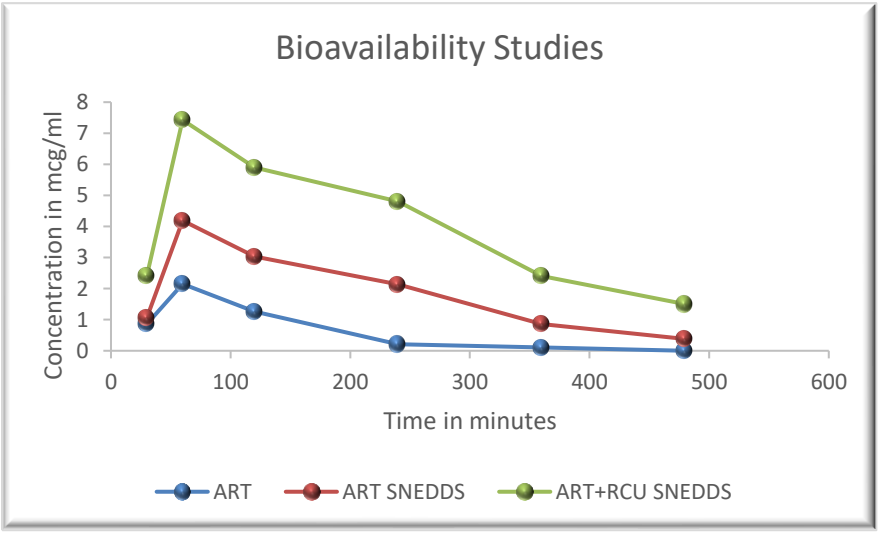


Figure 5.15 *In-vivo* kinetic studies of ART suspension ART-SNEDDS and ART+RCU (S) SNEDDS at different time intervals

5.11 Acute and Sub-Acute Toxicity studies of SNEDDS

5.11.1 Single dose acute toxicity study: As a result, the test samples were not hazardous up to 2000 mg/kg. The results of single dose toxicity study are given in Table 5.18.

Table 5.18 Results of single dose toxicity studies of SNEDDS formulation

Parameters	**Group-I: Normal control**	**Group-II: ART SNEDDS**	**Group-III: ART+RCU (S) SNEDDS**
Body weight (g)	198±3.8	194±6.7	191±5.4
Food Intake (g)	25±2.2	24±2.1	27±1.9
Water Intake (mL)	109±6.5	109±6.3	108±7.2
Muscle strength using rota rod (seconds)	149±6.5	157±5.5	148±10
Locomotion using actophotometer	98±9.5	97±6.8	100±10
Pain reflex using Eddys Hot Plate (Seconds)	1.8±0.23	1.8±0.15	1.5±0.14
Behaviour	Normal	Normal	Normal
Mortality	No	No	No

All the results were expressed as mean±SEM and statistical analysis was carried out by one way ANOVA followed by Dunnett's Multiple Comparison Test, where ($P < 0.05$) is considered as significant as compared to Group I.

5.11.2 Repeated dose toxicity study for 28 days:

In repeated dose toxicity study the test samples were administered to the test group for 28 days. The results are expressed in following table 5.28. The results of biochemical estimation in all the groups are clearly indicating that there was no significant change in any of the parameter as compare to normal group.

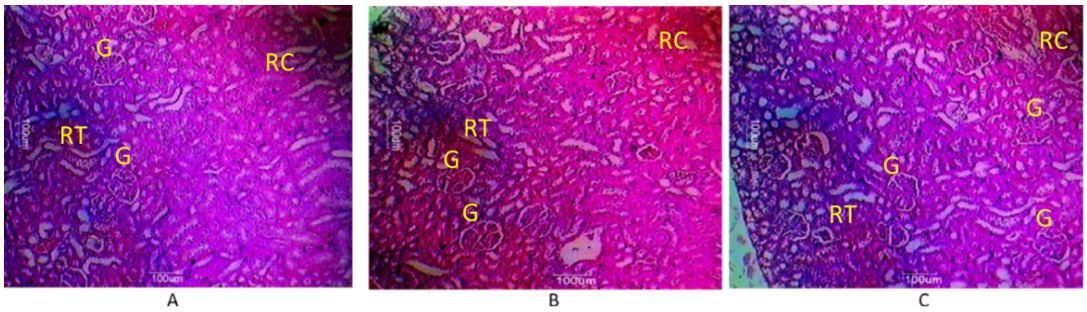
Table 5.19 Results of repeated dose toxicity studies of snedds formulations

Body parameters			
Parameters	Group-I	Group-II	Group-III
Body weight in gram	237±5.3	240±4.1	241±3.7
Locomotor activity	91±6.34	87±8,23	93±23-02
Hematological parameters			
RBC $x10^6$/µl	7.5±0.22	7.4±0.13	7.9±0.20
WBC $x10^3$/µl	8.2±0.55	8.1±0.42	8.4±0.34
Platelet $x10^3$/µl	685±57	708±55	742±57
Haemoglobin in g/dl	16±0.75	14±0.74	15±0.62
Total Cholesterol mg/dl	86±3.8	87±4.7	89±3.4
Triglycerides mg/dl	75±3.3	76±4.9	79±3.5
HDL mg/dl	27±2.6	30±2.8	29±2.6
LDL mg/dl	59±3.6	57±3.9	60±1.8
VLDL mg/dl	15±0.65	15±0.99	16±0.70
Blood Glucose mg/dl	93±3.5	86±3.3	91±4.3
Liver function test parameters			
SGOT IU	31±3.5	36±3.0	31±3.1
SGPT IU	43±4.6	39±5.2	39±3.6
Total Bilirubin mg/dl	1.2±0.12	1.0±0.14	1.0±0.13
Kidney function test parameters			
Blood Urea Nitrogen mg/dl	13±1.1	13±1.2	13±1.1
Uric Acid mg/dl	2.4±0.15	2.0±0.19	1.9±0.19
Creatinine mg/dl	1.9±0.21	1.8±0.22	1.6±0.24

All the results were expressed as mean±SEM and statistical analysis was carried out by one way ANOVA followed by Dunnett's Multiple Comparison Test, where ($P < 0.05$) is considered as significant as compared to Group I.

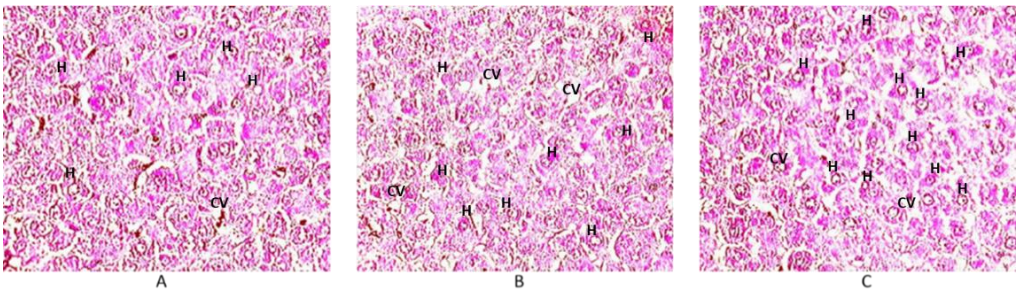
5.11.2.1 Histological evaluation:

The renal tissues as well as hepatocytes are normal and intact as compare to normal group. Histology of Kidney and Liver are depicted in Figure 5.16 and 5.17 respectively.



RC: Normal Renal Capsule, RT: Normal Renal Tubule, G: Normal Glomeruli

Figure 5.16 Histology of renal tissues (H&E staining, 40X) after 28 days of treatment of test sample, A: Group I, B: Group II, C: Group III



CV: Normal Central Vein; H: Normal Hepatocytes

Figure 5.17 Histology of liver tissues (H&E staining, 40X) after 28 days of treatment of test sample, A: Group I, B: Group II, C: Group III

Where,

A- represented Normal control Group I

B- represented ART loaded SNEDDS Group-II (2000 mg/kg per oral)

C- represented ART+RCU (S) SNEDDS Group-III (2000 mg/kg per oral)

This confirms that the test samples at administered dose are safe and not causing any structural damage to the renal and hepatic systems.

5.12 MTT Assay of test compound: MTT assay was performed using the J774 macrophage cell culture and the results shown in Figure 5.18, treated groups' optical densities (OD) significantly (P<0.001) increased when compared to the OD of the control (Tab 5.20).

Table 5.20 MTT Assay

Concentration ug/ml	Mean±SEM
Normal	0.83±0.0082
Control	0.33±0.0066
ART-SNEDDS 5 ug/ml	0.71±0.028
ART-SNEDDS 10 ug/ml	0.77±0.0055
ART-SNEDDS 20ug/ml	0.73±0.038
ART+RCU-SNEDDS 5 ug/ml	0.71±0.042
ART+RCU-SNEDDS 10 ug/ml	0.68±0.033
ART+RCU-SNEDDS 20ug/ml	0.75±0.0078
ART 5 ug/ml	0.63±0.018
ART 10 ug/ml	0.61±0.047
ART 20ug/ml	0.59±0.034

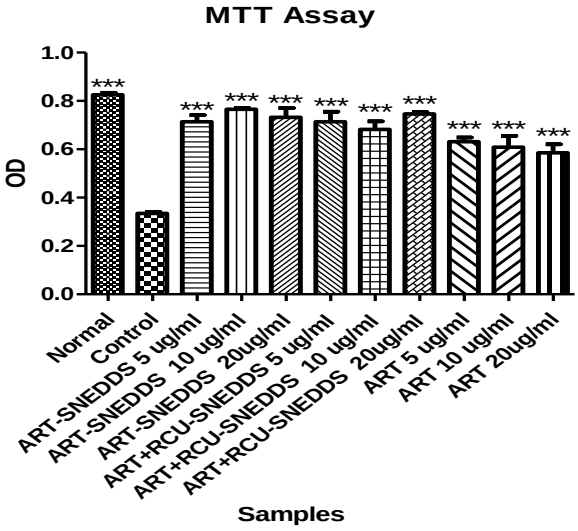


Figure 5.18 MTT Assay

All the results were expressed as mean±SEM and statistical analysis was carried out by one way ANOVA followed by Dunnett's Multiple Comparison Test, where (P < 0.05) is considered as significant as compared to the Normal group.

We can conclude that our formulation has normal cell protection properties that should be exploited for human benefit.

5.13 Haemolytic toxicity

To estimate the toxicity following intravenous administration, *ex-vivo* haemolytic toxicity research on plain ART, ART SNEDDS, and ART+RCU (S) SNEDDS was carried out. (Figure 5.19). The percentage of hemolysis, and statistical analysis determined that the difference from the control group was significant (P <0.001).

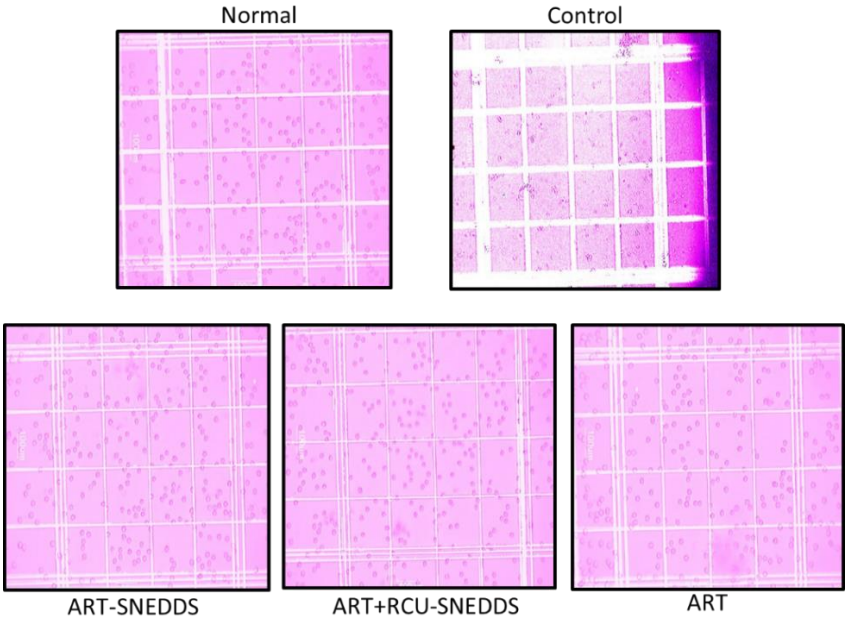


Figure 5.19 Haemolytic studies

Table 5.21: Assessment of Haemolytic activity of ART, ART-SNEDDS, and ART+RCU-SNEDDS

Group	**Mean±SEM**
Control	0.95±0.0063
Vehicle Control	0.05±0.037
ART 2µg/mL	0.04±0.023
ART 4µg/mL	0.01±0.0033
ART 8µg/mL	0.07±0.038
ART-SNEDDS 2µg/mL	0.01±0.0010
ART-SNEDDS 4µg/mL	0.05±0.026
ART-SNEDDS 8µg/mL	0.03±0.019
ART+RCU-SNEDDS 2µg/mL	0.01±0.0023
ART+RCU-SNEDDS 4µg/mL	0.03±0.015
ART+RCU-SNEDDS 8µg/mL	0.01±0.0012

Table 5.22: % Haemolysis of ART, ART-SNEDDS, and ART+RCU-SNEDDS

S/No.	Groups	% Haemolysis
1	Control	100.0
2	Vehicle Control	5.8
3	ART 2µg/mL	4.8
4	ART 4µg/mL	1.9
5	ART 8µg/mL	7.9
6	ART-SNEDDS 2µg/mL	1.9
7	ART-SNEDDS 4µg/mL	5.8
8	ART-SNEDDS 8µg/mL	3.5
9	ART+RCU-SNEDDS 2µg/mL	1.8
10	ART+RCU-SNEDDS 4µg/mL	3.2
11	ART+RCU-SNEDDS 8µg/mL	1.5

All the results were expressed as mean±SEM and statistical analysis was carried out by one way ANOVA followed by Dunnett's Multiple Comparison Test, where ($P < 0.05$) is considered as significant as compared to control group.

5.14 *In-vitro* and *In-vivo* anti-malarial activity studies of ART SNEDDS and ART+RCU(S) SNEDDS

5.14.1 *In-vitro* Antiplasmodial activity of ART SNEDDS and ART+RCU(S) SNEDDS: Among the two samples, ART+RCU(S) SNEDDS showed the strongest antiplasmodial activity with IC_{50} value of 393±8.2** µM as shown in the figure 5.20 and table 5.23.

Table 5.23 Concentration and IC_{50} values of *In-vitro* Antiplasmodial activity of ART, ART SNEDDS and ART+RCU(S) SNEDDS

IC50 in µM	ART	ART+SNEDDS	ART+RCU SNEDDS
IC50 I	462.50	410.15	377.71
IC50 II	486.34	430.89	405.82
IC50 III	450.76	412.20	395.37
Mean±SEM	467±10	418±6.6*	393±8.2**

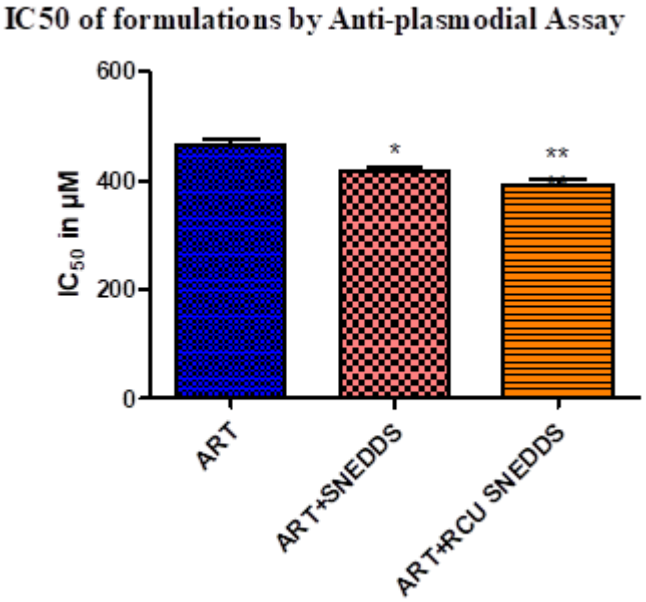


Figure 5.20 IC_{50} values of *In-vitro* Antiplasmodial activity of ART, ART SNEDDS and ART+RCU(S) SNEDDS

All the results were expressed as mean±SEM and statistical analysis was carried out by one way ANOVA followed by Dunnett's Multiple Comparison Test, where ($P < 0.05$) is considered as significant as compared to Group I.

The CQ-sensitive *Plasmodium falciparum* MRC-2 strain is resistant to both ART SNEDDS and ART+RCU SNEDDS in *In-vitro*, however ART+RCU SNEDDS exhibits significantly better results in IC_{50} [66].

5.14.2 *In-vivo* anti-malarial studies

Effect on Parasitemia and Survival Time

Both the samples showed promising anti-plasmodial activity against D6 *Plasmodium falciparum* showing the % parasitemia 7.8 and 10.4 for ART+RCU SNEDDS and ART SNEDDS respectively as shown in the figure 5.21 and Table 5.24.

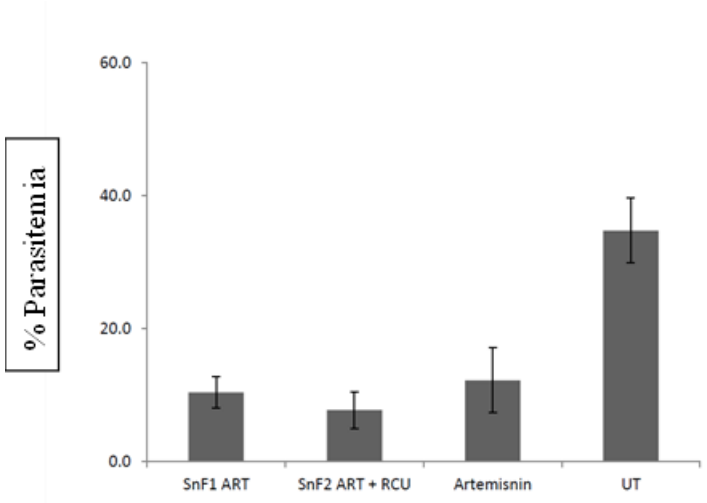


Figure 5.21 Graphs showing % Parasitemia in *In-vivo* anti-plasmodial studies of ART SNEDDS and ART+RCU(S) SNEDDS

Table 5.24 %Paracetemia in *In-vivo* antiplasmodial studies of ART SNEDDS and ART+RCU(S) SNEDDS

Groups	1	2	3	4	5	6	Average	% Parasitemia	STDEV	SEM
SnF1 ART	44	53	51	55	54	56	52.2	10.4	4.0	2.3
SnF2 ART+RCU	38	42	46	31	35	41	38.8	7.8	4.7	2.7
ART	56	65	48	57	69	72	61.2	12.2	8.5	4.9
Control (Untreated)	166	174	183	168	171	181	173.8	34.8	8.5	4.9
RBC Counted	500									

The SnF2 i.e., ART+RCU SNEDDS shown synergistic effect probably due to enhancing the bioavailability of the ART as well as it might be reduced the metabolism of ART by inhibiting the metabolizing enzyme CYP2C19. This might have increased the stay of ART in the body resulting in to increased antiparasitic effect.

6. Chapter: Summary & Conclusion

With a high likelihood of developing resistance to the majority of traditional and widely used anti-malarial drugs, malaria remained a significant worldwide health issue. Artemisinin-Based Combination Therapy (ACT) is the first-line therapy now advised by the WHO for the majority of malaria infections.

However, according to recent findings, resistance has emerged to both artemisinin and its derivatives. This has increased the level of worry regarding the use of artemisinin for the treatment of malaria. This is one of the most serious reasons why malaria control efforts have failed.

Therefore, to overcome artemisinin's drawbacks, either new antimalarial medications should be investigated or bio-enhancers should be used with artemisinin. It is effective against nearly all *Plasmodium species*, even resistant *P. falciparum*, However, full clearance of this medication is slow due to factors such as low half-life, Biopharmaceutics Classification System (BCS) class solubility, slow clearance, reinfection with parasites, limited bioavailability, etc. As a monotherapy, artemisinin is not advised. And hence, finding a natural component like Cow urine as a potent and appropriate bio-enhancer for artemisinin was believed to make a remarkable significance in the therapeutic management of malaria.

Combining ART with Cow urine could able to promote the anti-malarial effect and further, it can reduce the cost of converting ART into their derivatives. Moreover, reduction of size to the nano range is one of the best methods to increase the solubility. Moreover, lipid-based formulations provide increased absorption and increased bio-availability thereby.

Thus, the attempts have been made to develop SNEDDS formulation which adds novelty to our research work. ART-SNEDDS and ART+RCU(S) SNEDDS were designed to have a higher rate of dissolution and bioavailability. In the current study, we were able

to develop an optimized ART-SNEDDS and ART+RCU(S) SNEDDS formulation that outperforms ART suspension in terms of quality.

Furthermore, CYP2B6, CYP3A4, and CYP2A6 all metabolize artemisinin. Artemisinin enhances its own elimination (autoinduction) by inducing CYP2C19 and other cytochromes involved in its own metabolism. For the treatment of malaria, the oral artemisinin dosage is 250 mg daily for the first nine days and 500 mg on the tenth day. Additionally, monotherapy is ineffective because the condition returns after seven days.

Thus, artemisinin is always administered in combination therapy together with other medications. In this regard, our formulation's ability to reduce infection and improve drug absorption was deemed promising. Additionally, it was discovered that when compared to ART pure, the dose's efficacy was boosted twofold. It is evident that ART+RCU (S) SNEDDS, when administered with a dose decrease of twofold and a regimen reduction from ten to five days, can be one of the most successful combination medication therapies for the treatment of malaria

7. Chapter Future Perspective

The current study may be able to provide a solution for Artemisinin monotherapy and Artemisinin Combination Therapy resistance by introducing an inexpensive natural ingredient Cow Urine Residue (RCU) that was able to boost the bioavailability and efficacy of ART when given in the form of SNEDDS. The findings of this study lend support to researchers' claims that cow urine and its derivatives are excellent therapies for a number of ailments and infections. As a result, it is recommended that *in-vivo* efficacy testing be performed using the formfallation. Additional studies should be conducted to isolate and analyze compounds discovered in the RCU for anti-plasmodial activity. Further study is needed to establish a better understanding of the mechanisms of action and molecular modifications that support these findings.

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